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FULL PAPER

Temperature-dependent phosphate solubilization by cold- and pH-tolerant species of *Aspergillus* isolated from Himalayan soil

K. Rinu · Anita Pandey

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Abstract Ten species of *Aspergillus* isolated from soil samples collected from different locations in the Indian Himalayan region have been studied for their growth requirements and tricalcium phosphate solubilization at different temperatures. The *Aspergillus* species could grow at low temperature and tolerated a wide range of pH. Phosphate solubilization by various *Aspergillus* species ranged between 374 μg/ml (*A. candidus*) to 1394 μg/ml (*A. niger*) at 28°C, 33 μg/ml (*A. fumigatus*) to 2354 μg/ml (*A. niger*) at 21°C, 93 μg/ml (*A. fumigatus*) to 1452 μg/ml (*A. niger*) at 14°C, and 21 μg/ml (*A. wentii*) to 83 μg/ml (*A. niger*) at 9°C. At 21 and 28°C, phosphate solubilization showed a decrease within 4 weeks of incubation, whereas at 9°C and 14°C, it continued further up to 6 weeks of incubation. In general, phosphate solubilization by different *Aspergillus* species was recorded at a maximum of 28°C or 21°C; biomass production was favored at 21°C or 14°C. Conversely, *A. nidulans* and *A. sydowii* exhibited maximum phosphate solubilization at 14°C and produced maximum biomass at 21°C. Data suggest that suboptimal conditions (higher or lower temperature) for fungal growth and biomass production were optimal for the production of metabolites involved in phosphate solubilization. Significant negative correlations were obtained between pH and phosphate solubilization for eight species at 28°C, for seven at 21°C, and for nine at 14°C. Extracellular phosphatase activity was exhibited only in case of *A. niger*, whreas intracellular phosphatase activity was detected in all species, the maximum being in *A. niger*. Statistically significant positive or negative correlations were obtained between phosphate solubilization and other parameters in most cases at different temperatures.

Keywords *Aspergillus* spp. · pH tolerance · Cold tolerance · Phosphate solubilization · Phosphatase · Indian Himalayan region

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Introduction

Microorganisms play a fundamental role in the biogeochemical cycling of phosphorus in natural ecosystems. As phosphate solubilization is a prime process for plant growth, the importance of phosphate solubilizing microorganisms is well recognized (Velazquez and Rodriguez-Barrueco 2007). Temperature, pH, and biomass are vital factors for various activities of microorganisms. The major microbiological process by which insoluble phosphorus compounds are mobilized is by production of organic acids. Fungi and bacteria release organic acids such as citric, gluconic, and ketogluconic to liberate phosphates in to the soil (Sperber 1958; Cunningham and Kuiack 1992; Goldstein 1995; Wahid and Mehana 2000). Besides organic acid production, release of protons accompanying respiration or ammonium (NH_4) assimilation also contributes to phosphate solubilization (Illmer and Schinner 1992). Phosphatases and phytases are known to play an important role in phosphate solubilization through catalyzing the hydrolysis of phosphatic compounds (Michael and Robert 1984; Tarafdar and Jungk 1987).

Diversity and dominance of a microbial community depend upon environmental factors, both climatic and edaphic. Members of the genus *Aspergillus* have been of interest because of their positive as well as negative

impacts. This has led to an increased need to understand the occurrence and distribution of these fungi in various geographical locations in nature (Klich 2002). Literature on microbial diversity of colder regions, such as the Indian Himalayan region (IHR), is scanty. Therefore, various studies on microbial diversity of the IHR with reference to their potential applications have been initiated in our laboratory. A number of fungal genera, *Aspergillus* and *Penicillium* in particular, survive and dominate in acidic and low-temperature areas of the IHR (Pandey et al. 2008). The aim of this study was to determine the phosphate solubilization potential of aspergilli isolated from Himalayan soil. These investigations were carried out on ten species of *Aspergillus* at different temperatures. Correlations were developed between phosphate solubilization potential and associated factors, namely, pH, biomass production, and phosphatase activity.

Materials and methods

Aspergillus cultures

Cultures of *Aspergillus* species were taken from the culture collection developed in the laboratory. Cultures were originally isolated from soil samples collected from different locations in the IHR. All species were maintained on agar slants at 4°C. Nine *Aspergillus* species belonged to the forest sites (*A. candidus* and *A. nidulans* to *Betula* forest soil; *A deflectus*, *A. fumigatus*, and *A. niger* to Cedrus-Taxus forest soils; *A flavus*, *A. glaucus*, and *A. wentii* to rhododendron forest soil; *A sydowii* to oak forest soil), whereas *A. parasiticus* was an isolate from tea rhizosphere soil. The study site covered a wide altitudinal range between 1800 and 3610 m above sea level, representing temperate to alpine climatic conditions of the IHR. Whereas the soil pH at sampling sites ranged between 4.5 and 6.5, all locations experience heavy rainfall and snowfall, maintaining low temperature up to subzero levels (Pandey and Palni 2007). The mean monthly temperature of the locations lies between 5.5°C (January) and 20.1°C (August). Therefore, the experiments were carried out considering a range of temperatures suitable for the growth of mesophiles and psychrotrophs.

Temperature, pH, and salt tolerance of *Aspergillus* cultures

Cultures were checked for their minimum, optimum, and maximum growth at different temperatures (4, 9, 14, 21, 28, 35, 42, and 55°C) and pH (1–13 at an interval of 1), and for tolerance of sodium chloride (NaCl) (5, 7, 10, 12 and 15%), using potato dextrose agar (PDA). Growth was recorded up to day 28 at weekly interval. A minimum of 5 mm per colony on the agar plate was considered as growth.

Estimation of phosphate solubilization, biomass, and pH

Pikovskaya's agar plates (Pikovskaya 1948) were inoculated individually with different species of *Aspergillus* at 4, 9, 14, 21, and 28°C. Plates were observed for zone of solubilization around the colony up to day 42 of growth at weekly intervals. Zone of solubilization was calculated by subtracting the total diameter of the colony from the colony diameter plus zone of clearance. Cultures were tested for their phosphate solubilization efficiency quantitatively in Pikovskaya's broth containing tricalcium phosphate (TCP) (0.5 µg/100 ml); 0.5 g of TCP was weighed into a 250-ml Erlenmeyer's flask, and 100 ml of Pikovskaya's broth without phosphorus source was poured in to it. The initial pH of the media before autoclaving was 7.50. The autoclaved media was inoculated with a 5-mm disc of *Aspergillus* species and incubated at 9 and 14°C for 6 weeks and at 21 and 28°C for 4 weeks. In view of the slow growth and persistence of the desired activity at low temperature, incubation for an additional 2 weeks was considered. The experiments were conducted in static conditions until and unless stated. The culture filtrate was withdrawn from the respective flasks on every seventh day of incubation and filtered through Whatman No. 42 filter paper. The culture filtrate was then analyzed for phosphorus (P_2O_5) production using the chlorostannous-reduced molybdophosphoric acid blue method (Jackson 1967). Absorbance was taken at 700 nm using a Uvikon spectrophotometer (Kontron Instruments, UK). Culture filtrate pH (Systronics, India) was also recorded each time. Culture biomass was estimated on every seventh day of incubation from the same flasks that were used for the above experiments. The mycelium was collected after filtration of the broth culture and dried at 65°C for 72 h. Biomass detection was not possible at 9°C due to less growth.

Estimation of extracellular and intracellular phosphatase activity

Extracellular acidic and alkaline phosphatase activity was estimated at weekly intervals from Pikovskaya's broth culture incubated at different temperatures (9, 14, 21, and 28°C). Intracellular acidic and alkaline phosphatase activity was done at 21°C only. The culture filtrate was withdrawn and filtered through Whatman No. 42 filter paper at 4°C. The filtrate was used to estimate extracellular acidic and alkaline phosphatase activity. The mycelia was collected and washed 10 times with sterile distilled water and macerated in 0.02 M Tris buffer (1:1 w/v, pH 7.5). Then, the macerate was centrifuged at 16000*g* for 20 min (Hitachi, Himac CR 22G/Rotor,

R20A2) at 4°C and the supernatant was collected. This supernatant was used for enzyme assay. Estimation of acidic and alkaline phosphatase was performed following the methods given in our previous report (Pandey et al. 2008). One enzyme unit (U) was defined as the amount of enzyme that catalyzed the formation of 1 μM of end product (*p*-nitrophenol) in 1 min under experimental conditions (Tabatabai and Bremner 1969). All experiments were done in triplicate and repeated twice. Glassware used was treated with 2 normal hydrochloric acid (2N HCl) to make it free of detergents and other contaminants.

Statistical analysis

Data were analyzed with the computer program Excel (Microsoft Corp.) for graphical representations, mean values, and standard deviations (SDs). The correlations (partial) between phosphate solubilization and pH changes, biomass production, and phosphatase were calculated using the computer software SPSS (Statistical Package for Social Sciences)/PC (1986).

Results

Growth characteristics of *Aspergillus* species

Results on temperature and pH requirement of 10 *Aspergillus* species and their salt tolerance are presented in Table 1. The optimum growth of various *Aspergillus* species was recorded at 21 or 28°C; the cultures tolerated maximum temperature up to 35 or 42°C following 1 week of incubation. The minimum temperature requirement was recorded between 4 and 9°C on prolonged incubation period. The pH tolerance of all species ranged between 2 and 12, with the optimum being between 7 and 9. Tolerance for salt concentration ranged between 12 and 15% in the medium.

Qualitative estimation of phosphate solubilization on Pikovskaya's Agar

The qualitative estimation of phosphate solubilization on Pikovskaya's agar showed the maximum zone of solubilization by *A. niger*, followed by *A. sydowii*, *A. deflectus*, *A. candidus*, *A. nidulans,* and *A. glaucus*. *A. wentii* and *A. flavus* produced yellow diffusible pigment around the colony at 28, 21, and 14°C on the Pikovskaya's agar plates, hence the zone was not visible. However, these species produced no pigment at 9°C and formed a 1-mm zone of solubilization around the colony. *A. parasiticus* and *A. fumigatus* exhibited no solubilization zone at any temperature. At 4°C, solubilization growth and zone was absent in all cultures except *A. candidus*, which showed growth initiation and produced a narrow solubilization zone after 4 weeks of incubation.

Phosphate solubilization, biomass production, pH change, and phosphatase activity of *Aspergillus* species at different temperatures

Out of ten *Aspergillus* species studied, five species, namely, *A. candidus* (7.48% on day 21), *A. deflectus*

Table 1 Temperature, pH, and salt tolerance of ten species of *Aspergillus*

Aspergillus species	Temperature tolerance (°C)			pH tolerance			Salt tolerance (%)	Accession no.
	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum		
A. candidus	4[d]	21[a]	42[a]	2[c]	7[c]	12[c]	12	ARIFCC774
A. deflectus	9[c]	28[a]	42[a]	3[c]	8[c]	12[c]	12	ITCC5016
A. flavus	9[c]	28[a]	35[a]	2[c]	7[c]	12[c]	12	ARIFCC1161
A. fumigatus	9[c]	21[a]	42[a]	2[c]	7[c]	12[c]	15	ITCC3717
A. glaucus	9[b]	21[a]	42[a]	2[c]	7[c]	12[c]	12	ARIFCC771
A. nidulans	9[b]	28[a]	42[a]	2[c]	7[c]	12[c]	15	ARIFCC772
A. niger	9[b]	28[a]	42[a]	2[c]	9[c]	12[c]	15	ITCC2546
A. parasiticus	4[b]	28[a]	42[a]	2[c]	7[c]	12[c]	12	ITCC4239
A. sydowii	9[b]	28[a]	35[a]	2[c]	9[c]	12[c]	15	ITCC4210
A. wentii	9[c]	28[a]	42[a]	2[c]	8[c]	12[c]	12	ARIFCC773

ARIFCC Agharkar Research Institute Fungal Culture Collection, Pune, India, *ITCC* Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India

[a] Day 7

[b] Day 14

[c] Day 21

[d] Day 28

(9.68% on day 28), *A. flavus* (18.28% on day 14), *A. parasiticus* (7.88% on day 21), and *A. wentii* (9.24% on day 28) exhibited maximum activity at 28°C (Table 2). Interestingly, biomass production by these cultures was less at this temperature, even less than that recorded at 14°C, for *A. candidus*, *A. nidulans*, and *A. niger*. Values for biomass production and phosphate solubilization were significantly ($P \leq 0.01$ or ≤ 0.05) correlated in eight cultures. Maximum pH decrease (2.96) was recorded in *A. niger* at 28°C on day 7 of incubation. The pH decrease at 28°C was significantly correlated with phosphate solubilization in *A. candidus*, *A. fumigatus*, *A. parasiticus*, and *A. sydowii* ($P \leq 0.01$) and in *A. deflectus*, *A. niger,* and *A. wentii* ($P \leq 0.05$). Extracellular phosphatase activity was detected only in *A. niger*, which was maximum (31.66 U of acidic) on day 21 and (0.36 U of alkaline) on day 14 of incubation at 28°C (Fig. 1b).

At 21°C, *A. niger* (47.00% on day 7) and *A. glaucus* (15.56% on day 28) solubilized maximum supplied TCP in Pikovskaya's broth (Table 2). In *A. glaucus* and *A. sydowii* (11.92% on day 28), phosphate solubilization continued to increase even after day 28 of incubation. Biomass production was significantly correlated with phosphate solubilization in *A. fumigatus*, *A. glaucus*, *A. nidulans*, *A. parasiticus,* and *A. wentii*. A negative correlation ($r = -0.608$, statistically not significant) was obtained between phosphate solubilization and biomass produced by *A. niger*; 21°C was found to be an appropriate temperature for biomass production in all ten *Aspergillus* species except *A. parasiticus*. However, phosphate solubilization was comparatively less at this temperature in all cultures except *A. niger* and *A. glaucus*. The optimum temperature for growth of these cultures was 21°C, which was not optimum for phosphate solubilization. The persistence of phosphate solubilization after day 28 in *A. glaucus* and *A. sydowii* was significantly ($P \leq 0.01$) correlated with decrease in pH. Decrease in pH was significantly correlated with phosphate solubilization in all cultures except *A. deflectus* (2.18% on day 28), *A. fumigatus* (0.66% on day 28), and *A. wentii* (1.78% on days 14 and 21). Maximum extracellular acidic and alkaline phosphatase activity of *A. niger* was recorded at this temperature (Fig. 1a). At 21°C, extracellular acidic phosphatase was estimated at 21 U on day 7, which became constant on further incubation. Extracellular alkaline phosphatase was recorded as 4 U on day 7, which decreased up to day 28. There was a significant correlation ($P \leq 0.01$) between phosphate solubilization and decrease in pH in acidic and alkaline phosphatase activity of *A. niger* at 21°C. The change in pH was significantly correlated with alkaline phosphatase activity. Intracellular acidic and alkaline phosphatase activity was estimated in all *Aspergillus* species (Fig. 2). *A. niger* showed maximum activity of acidic phosphatase (37.7 U on day 7) among the ten species. Conversely, recorded alkaline phosphatase was very low (3 U on day 14) for *A. niger*. *A. flavus* showed 19.33 U activity of acidic phosphatase on day 21 of incubation.

At 14°C, phosphate solubilization persisted during prolonged incubation, even after day 42 in five species (Tables 2, 3). It was maximum in *A. niger* (29.04% on day 35), followed by *A. flavus* (15.48% on day 21), *A. glaucus* (15.40% on day 42), *A. sydowii* (14.6% on day 42), *A. nidulans* (9.14% on day 28), *A. wentii* (8.14% on day 35), *A. deflectus* (6.04% on day 42), *A. parasiticus* (3.8% on day 42), *A. candidus* (2.3% on day 42), and *A. fumigatus* (1.86% on day 35). *A. nidulans* and *A. sydowii* gave maximum phosphate solubilization at 14°C. Biomass production was also found significantly correlated with phosphate solubilization in all species except *A. flavus*, *A. fumigatus*, and *A. nidulans*. The decrease in pH was found to be much less in *A. candidus*, *A. fumigatus*, and *A. parasiticus* at 14°C (Tables 2, 3). In other species, the order of pH reduction was *A. nidulans* < *A.wentii* < *A. deflectus* < *A. flavus* < *A. sydowii* < *A. glaucus* < *A. niger*. The decrease in pH was significantly correlated with phosphate solubilization in all species except *A. glaucus* when analyzed for the entire incubation period. In *A. niger*, the pH of the culture media was recorded as 1.52 on day 42 of incubation. Extracellular enzyme activity was estimated as almost negligible in all species at 14°C. *A. parasiticus*, *A. deflectus*, *A. sydowii*, *A. glaucus,* and *A. candidus* showed persistence of phosphate solubilization, even up to day 42 of incubation.

At 9°C, growth was much less and biomass production could not be detected in any *Aspergillus* species. Likewise, phosphate solubilization, reduced pH, and extracellular phosphatase activity were also recorded to be less at this temperature (Tables 2, 3).

Discussion

Although *Aspergillus* species in this study were originally isolated from acidic soils of higher altitudes of the IHR, they exhibited tolerance for extreme pH ranging from 2 to 12. Similarly, the species also showed tolerance to a wide temperature range between 4 and 9°C (minimum) and 35 and 42°C (maximum). This indicated the dominance of the species, which possess the ability to tolerate and survive under a wide range of environmental conditions, such as low temperature and acidic soils. The species, therefore, can be regarded as temperature and acid tolerant rather than true psychrophiles or acidophiles, respectively. Such soils have been reported for dominance of cold- and acid-tolerant organisms, both by bacteria and fungi (Pandey et al. 2006a, b). Eight species of *Penicillium*

Table 2 Phosphate solubilization and changes in pH and biomass (dry weight basis) of *Aspergillus* spp.

Aspergiilus species	Temperature (°C)	Phosphate solubilization (μg/ml)				pH				Biomass (mg)			
		Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
A. candidus	9	0	4	14	22	7.42	7.35	7.69	7.53	ND	ND	ND	ND
	14[c]	32	54	74	94	7.38	7.7	7.48	7.41	ND	90	200	270
	21[a]	12	76	99	70	7.27	6.82	6.17	6.95	340	390	420	550
	28[c]	216	274	374	299	7.06	6.96	5.0	5.45	58	80	150	180
A. deflectus	9	0	8	14	23	7.36	7.53	7.62	7.60	ND	ND	ND	ND
	14[c]	0	16	43	84	7.49	6.67	6.12	5.59	ND	50	160	200
	21	45	75	89	109	5.56	5.25	4.93	5.33	430	530	680	360
	28[c]	13	148	240	484	6.27	5.52	4.72	5.16	280	320	400	370
A. flavus	9	0	4	13	18	7.45	7.53	7.73	7.53	ND	ND	ND	ND
	14[a]	62	67	673	774	7.3	6.32	5.68	5.11	160	220	250	270
	21[a]	14	32	100	100	5.27	4.64	4.40	4.52	380	520	640	440
	28	654	914	864	754	4.3	3.96	4.35	4.96	410	427	460	470
A. fumigatus	9	0	5	15	22	7.34	7.54	7.43	7.48	ND	ND	ND	ND
	14[a]	0	15	30	55	7.45	7.75	7.55	7.72	140	230	270	300
	21[b]	11	19	32	33	5.64	5.85	5.43	5.23	350	470	520	460
	28[c]	35	153	200	530	7.01	6.98	5.59	5.09	30	120	170	450
A. glaucus	9	0	4	10	20	7.34	7.53	7.70	7.51	ND	ND	ND	ND
	14[b]	94	234	440	610	6.58	4.52	3.88	3.68	130	170	250	290
	21[c]	112	384	594	773	6.39	4.99	4.89	4.96	370	590	670	780
	28[b]	190	470	448	695	4.3	4.08	4.62	4.67	340	400	410	450
A. nidulans	9	0	4	18	28	7.30	7.51	7.73	7.51	ND	ND	ND	ND
	14[a]	91	174	239	457	7.06	5.60	5.50	5.86	150	280	370	440
	21[c]	16	82	86	78	6.62	5.00	5.42	6.12	360	520	620	630
	28[b]	217	238	356	426	4.52	4.68	4.85	6.21	210	290	360	320
A. niger	9	0	8	18	22	7.00	7.56	7.62	7.46	ND	ND	ND	ND
	14[c]	554	794	1220	1394	5.38	3.14	2.56	1.63	130	240	330	410
	21[a]	2354	2209	1701	1208	2.99	3.05	3.04	3.34	330	370	470	400
	28[a]	1394	1385	1284	1027	2.96	3.91	4.04	4.18	300	380	380	370
A. parasiticus	9	0	8	14	26	7.38	7.46	7.68	7.43	ND	ND	ND	ND
	14[c]	32	52	74	95	7.22	7.67	7.47	6.94	ND	ND	90	14
	21[c]	11	35	39	51	5.35	5.28	4.42	4.19	200	380	490	530
	28[c]	192	236	394	346	6.28	5.38	5.20	5.14	200	530	450	230
A. sydowii	9	0	8	14	18	7.22	7.48	7.60	7.55	ND	ND	ND	ND
	14[c]	0	9	54	380	7.55	6.83	5.50	5.50	60	130	240	360
	21[a]	116	395	503	596	5.22	4.53	3.73	3.24	460	700	610	390
	28[c]	64	418	554	354	5.71	3.85	3.89	4.24	210	260	340	410
A. wentii	9	0	4	12	18	7.39	7.53	7.71	7.41	ND	ND	ND	ND
	14[c]	18	54	94	214	7.4	6.8	6.22	5.74	120	160	210	250
	21[b]	15	89	89	88	5.34	5.83	5.20	5.16	370	510	560	490
	28[c]	39	224	314	462	6.70	4.83	5.24	5.27	230	220	430	420

ND not detected

[a] Phosphate solubilization significantly correlated with pH

[b] Phosphate solubilization significantly correlated with biomass

[c] Phosphate solubilization significantly correlated with both pH and biomass

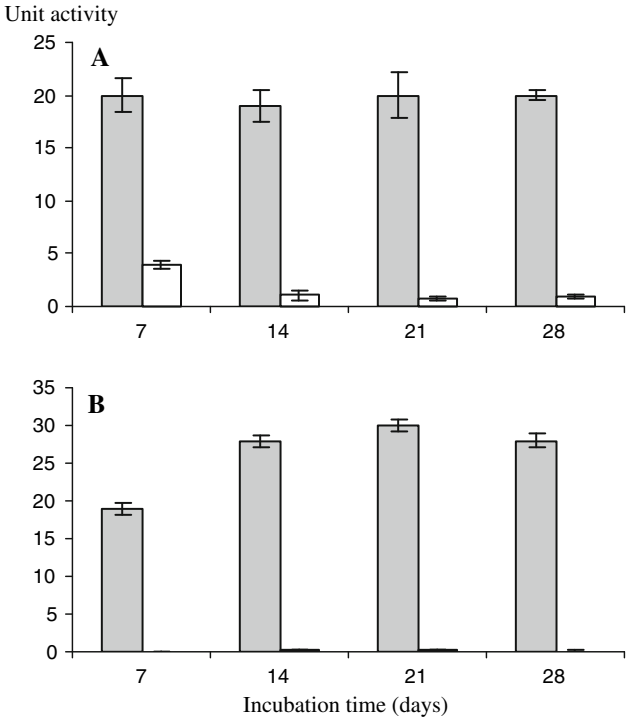

Fig. 1 Extracellular phosphatase enzyme activity of *Aspergiilus niger* at **a** 21°C and **b** 28°C [*filled bars* acidic, *open bars* alkaline, data are mean of three replicates (±standard deviation)]

(Pandey et al. 2008) and two of *Pseudomonas* (Pandey et al. 2002, Pandey et al. 2006a, b) of temperate origin have recently been reported for their phosphate solubilization potential. These species were also identified for their tolerance to a wide range of temperature and pH. Temperature and pH are important factors responsible for the occurrence of specific microbes in the soil.

Phosphate solubilization of *Aspergillus* species and the related parameters studied were found to be temperature dependent. The results obtained indicated that, in general, the optimal conditions for phosphate solubilization and biomass production differ. Illmer and Schinner (1992) reported higher phosphate solubilization of *Penicillium* species in suboptimal carbon-concentrated medium compared with that in optimum growth conditions. Barroso et al. (2006) reported maximum solubilization and biomass by *A. niger* at 30°C. Some species used in our study, such as *A. candidus* (176 µg/15 ml), *A. fumigatus* (212 µg/15 ml), and *A. niger* (30.60% P_2O_5), have earlier been reported for phosphate solubilization. These isolates originally belonged to tropical soils, and experiments were conducted at mesophilic temperature (Banik and Dey 1982). The phosphate solubilization potential of temperate cultures is higher in comparison with those of tropical origin.

Decrease in pH, due to production of organic acids such as citric acid, gluconic acid, oxalic acid, malic acid, etc., is known as one of the mechanisms responsible for phosphate solubilization (Salih et al. 1989; Cunningham and Kuiack 1992; Reyes et al. 2001). Organic acids can greatly increase the concentration of phosphate solubilization through chelation and exchange reaction (Gadd 1999). In our study, decrease in pH in all the *Aspergillus* species was found to be temperature dependent. Maximum decrease in pH was recorded at 21 and 28°C, coinciding with maximum phosphate solubilization. *A. nidulans* and *A. sydowii* showed maximum phosphate solubilization at 14°C. Less decrease in pH at 14°C coincided with low phosphate solubilization in *A. candidus*, *A. fumigatus,* and *A. parasiticus*. *A. niger* also showed minute activity at 9°C, indicating involvement of some other mechanisms. In higher temperatures (21 or 28°C), after achieving the maximum decrease in pH in 2–4 weeks of incubation, pH again increased without showing increase in phosphate solubilization, which may be due to cell death and lysis. However, in *A. niger* (at 21 and 14°C) and *A. glaucus* (at 28°C), phosphate concentration in the media also increased, probably due to cell lysis and phosphate liberation in the culture suspension. Similar observations on *Penicillium* species were reported by Illmer and Schinner (1992). They explained that the initial increase in phosphate concentration is due to acid production and later by altering the metabolism due to a lack of carbon, which may result in formation of an organophosphate compound. Consequently, due to alteration in the medium, cells may utilize this compound as an energy or nutrient source, resulting in a second release of phosphate.

Involvement of mechanisms other than acid production in phosphate solubilization has also been considered in some studies (Illmer et al. 1995; Richardson et al. 2000). Microorganisms, including fungi, are known to produce cold active metabolites at low temperature. Production of certain proteins at 8°C by *Metarhizium anisopliae* has been reported by De Croos and Bidochka (2001). These proteins, however, were not detected at 25°C. Phosphate-solubilizing microorganisms are known to produce phosphatases that are hydrolytic enzymes responsible for breakdown of insoluble compounds (Tarafdar et al. 2003). The experiments conducted on phosphatases resulted in production of intracellular enzymes in all species, whereas extracellular phosphatase activity was detected only in *A. niger*. The absence of extracellular phosphatase activity may be due to the availability of insoluble phosphorus in suspension culture (the experiments were conducted in Pikovskaya's medium containing 0.5% TCP). The effect of sublethal concentration of insoluble phosphate on phosphatase enzyme activity has been reported by Ramalingam and Prasanna (2006). Tarafdar et al. (2003) reported the production of extracellular phosphatases in Czapek dox broth (lacking insoluble phosphorus). Extracellular activity in

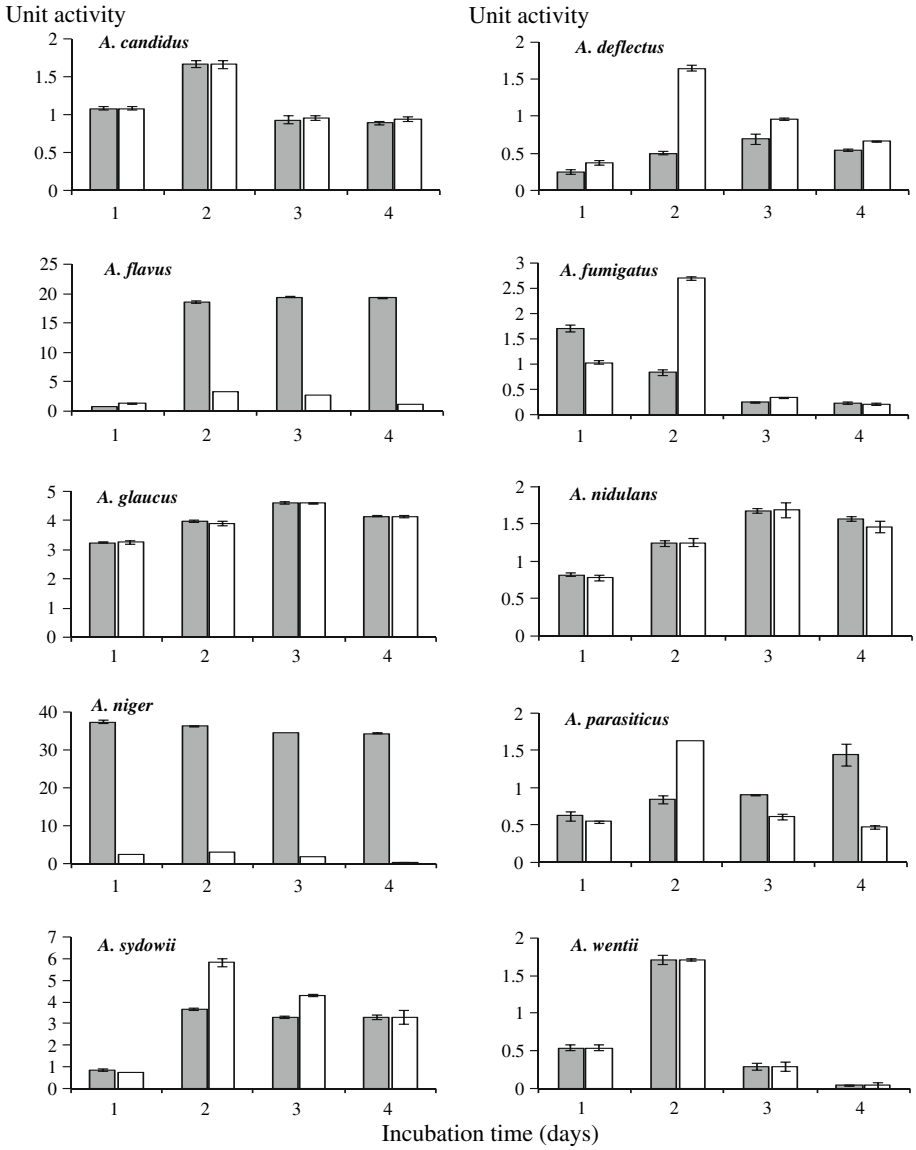

Fig. 2 Intracellular phosphatase activity of *Aspergillus* spp. at 21°C [*filled bars* acidic, *open bars* alkaline, data are mean of three replicates (±standard deviation)]

our study was probably not detected due to the presence of enough solubilized phosphorus in the medium due to fungal activity. Phosphatase is activated when there is low phosphorus availability. Aleksieva et al. (2003) reported approximately eight times higher phosphatase yield in phosphorus-deficient medium compared with phosphate-sufficient medium by the fungus *Humicola lutea*. Regulation of the production of two *A. ficcum* acid phosphatases by inorganic phosphate has been investigated by Shieh et al. (1969). However, this was not in tune with the results on *A. niger* in our study. Braibant and Content (2001) reported that the expression of *Mycobacterium bovis* phosphatase is not regulated by environmental inorganic phosphate concentration.

Intracellular phosphatases are also well known for the mineralization of phosphates (Gaur 1990). The increase in P concentration in the culture medium at the end of the incubation time (3–4 weeks) observed in many cases might be due to mineralization. Greater intracellular acidic phosphatase activity than alkaline of *A. niger* and *A. flavus* is probably due to the acidity in the medium. Intracellular acidic activity and alkaline phosphatase activity was found to be considerably greater in all cultures. Similar observations were reported by Tarafdar et al. (2003). The least extracellular alkaline phosphatase activity of *A. niger,* compared with its best extracellular acidic phosphatase activity, could obviously be explained with the maximum acidification of the culture medium.

Most studies reported earlier considered phosphate solubilization for a relatively shorter periods: 7 days (Wakelin et al. 2004), 14 days (Goenadi et al. 2000), 17 days (Vassileva et al. 1998), and 28 days (Wahid and Mehana 2000). We conducted our study up to 28 days at 28 and 21°C and up to 42 days at 9 and 14°C. Persistence of desired

Table 3 Phosphate solubilization and changes in pH and biomass (dry weight basis) of *Aspergillus* spp. at 9 and 14°C during 5th and 6th weeks of incubation at four different temperatures up to 4 weeks of incubation

Aspergillus species	Temperature (°C)	Phosphate solubilization (μg/ml)		pH		Biomass (mg)	
		Week 5	Week 6	Week 5	Week 6	Week 5	Week 6
A. candidus	9	33	24	7.53	7.29	ND	ND
	14	114	115	7.03	7.02	340	480
A. deflectus	9	23	15	7.45	7.41	ND	ND
	14	147	307	4.95	5.32	380	390
A. flavus	9	24	14	7.40	7.4	ND	ND
	14	402	392	5.34	5.75	310	500
A. fumigatus	9	32	25	7.58	7.55	ND	ND
	14	93	75	7.31	6.97	320	370
A. glaucus	9	23	20	7.29	7.46	ND	ND
	14	707	770	4.83	4.93	360	460
A. nidulans	9	24	20	7.47	7.33	ND	ND
	14	196	274	5.96	5.38	350	370
A. niger	9	66	83	7.35	7.38	ND	ND
	14	1452	1334	1.62	1.52	750	750
A. parasiticus	9	24	22	7.55	7.00	ND	ND
	14	147	194	7.48	6.42	170	250
A. sydowii	9	30	13	7.45	6.92	ND	ND
	14	407	730	5.37	5.48	440	410
A. wentii	9	21	14	7.45	7.62	ND	ND
	14	407	304	5.31	5.35	310	300

ND not detected

activity after a long incubation time, particularly at low temperatures, was envisaged considering the ecological significance under the temperate forest ecosystem. *A. niger* is the best reported fungal species for phosphate solubilization. In our study also, maximum solubilization (47%) of supplied phosphate was recorded with *A. niger* only, which is second (54%) to the earlier report by Omar (1998). Vassileva et al. (1998) reported 42% of solubilization of supplied phosphate in culture medium.

This is a preliminary study with a focus phosphate-solubilizing species of *Aspergillus* in temperate and alpine areas of the IHR. It is concluded that phosphate-solubilization potential of aspergilli is affected by environmental conditions, mainly temperature. Whereas production of organic acids by *Aspergillus* species is considered to play a major role in phosphate solubilization, involvement of phosphatases was also recognized. Investigations to identify and quantify organic acids and phytases are in progress. Production of pigments by some *Aspergillus* species was also temperature dependent. Variation in production of organic acids, enzymes, or pigments in response to different temperatures provides clues on survival of these fungi under temperate or alpine climatic conditions; this requires further investigation. Whereas most aspergilli are known to grow optimally at mesophilic temperatures, this study is important to provide insight into diversity and potential applications of *Aspergillus* species in colder areas of the IHR. Although *A. niger* revealed highest phosphate-solubilizing activity, other species with relatively lower activity are likely to play important roles in nutrient cycling under low temperature in the IHR. Efficient species could potentially be developed as "bioinoculants" for application such as enhancing biodegradation in colder mountainous regions. Fungal species with relatively low activity persisting for longer periods appear to be important for metabolic activities under low-temperature mountain ecosystems.

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References

Aleksieva P, Spasova D, Radoevska S (2003) Acid phosphatase distribution and localization in the fungus *Humicola lutea*. Z Naturforsch 58:239–243

Banik S, Dey BK (1982) Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate-solubilizing micro-organisms. Plant Soil 69:353–364

Barroso CB, Pereira GT, Nahas E (2006) Solubilization of $CaHPO_4$ and $AlPO_4$ by *Aspergillus niger* in culture media with different carbon and nitrogen sources. Braz J Microbiol 37:434–438

Braibant M, Content J (2001) The cell surface associated phosphatase activity of *Mycobacterium bovis* BCG is not regulated by environmental inorganic phosphate. FEMS Microbiol Lett 195:121–126

Cunningham JE, Kuiack C (1992) Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium billai*. Appl Environ Microbiol 52:1451–1458

De Croos JNA, Bidochka MJ (2001) Cold induced proteins in cold-active isolates of the insect-pathogenic fungus *Metarhizium anisopliae*. Mycol Res 105:868–873

Gadd GM (1999) Fungal production of citric and oxalic acid: Importance in metal speciation, physiology and biochemical processes. Adv Microb Physiol 41:47–92

Gaur AC (1990) Phosphate solubilizing microorganisms as biofertilizer. Omega scientific publishers, New Delhi

Goenadi DH, Siswanto, Sugiarto Y (2000) Bioactivation of poorly soluble phosphate rocks with a phosphorus-solubilizing fungus. Soil Sci Soc Am J 64:927–932

Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram-negative bacteria. Biol Agric Hortic 12:185–193

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol Biochem 24:389–395

Illmer P, Barbato A, Schinner F (1995) Solubilization of hardly-soluble $AlPO_4$ with P solubiling microorganisms. Soil Biol Biochem 27:265–270

Jackson ML (1967) Soil Chemical analysis. Prentice Hall of India, India

Klich MA (2002) Biogeography of *Aspergillus* species in soil and litter. Mycologia 94:21–27

Michael OG, Robert CE (1984) Fungal nutrition and physiology. Wiley, Canada

Omar SA (1998) The role of rock phosphate-solubilizing fungi and vesicular arbuscular-mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. World J Microbiol Biotechnol 14:211–218

Pandey A, Palni LMS (2007) The rhizosphere effect in the trees of the Indian Central Himalaya with special reference to altitude. Appl Ecol Environ Res 5:93–102

Pandey A, Palni LMS, Mulkalwar P, Nadeem M (2002) Effect of temperature on solubilization of tricalcium phosphate by *Pseudomonas corrugata*. J Sci Ind Res 61:457–460

Pandey A, Trivedi P, Kumar B, Palni LMS (2006a) Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Curr Microbiol 53(2):102–107

Pandey A, Trivedi P, Kumar B, Chaurasia B, Palni LMS (2006b) Soil microbial diversity from the Himalaya: need for documentation and conservation. National Biodiversity Authority Scientific Bulletin No. 5, Chennai, Tamil Nadu

Pandey A, Das N, Kumar B, Rinu K, Trivedi P (2008) Phosphate solubilization by *Penicillium* spp. isolated from soil samples of Indian Himalayan region. World J Microbiol Biotechnol 24:97–102

Pikovskaya RI (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya 17:362–370

Ramalingam N, Prasanna BG (2006) Effect of aluminum phosphate on alkaline phosphatase activity of polyurethane foam immobilized cyanobacteria. Curr Microbiol 53:194–197

Reyes I, Bernier L, Baziramakenga R, Antoun H (2001) Solubilization of phosphate rocks and minerals by a wild-type strain and two UV-induced mutants of *Penicillium rugulosum*. Soil Biol Biochem 33:1741–1747

Richardson A, Hadobas PA, Hayes JE (2000) Acid phosphomonoesterase and phytase activities of wheat (*Trticum aestivum* L) roots and utilization of organic phosphorus substrates by seedlings grown in sterile culture. Plant Cell Environ 23:397–405

Salih HM, Yahya AI, Abdul-Rahem AM, Munam BH (1989) Availability of phosphorus in a calcarious soil treated with rock phosphate or superphosphate as affected by phosphate dissolving fungi. Plant Soil 120:181–185

Shieh TR, Wodzinski RJ, Ware JH (1969) Regulation of the formation of acid phosphatases by inorganic phosphate in *Aspergillus ficcum*. J Bacteriol 100:1161–1165

Sperber JI (1958) Solution of apatite by microorganisms producing organic acids. Aust J Agric Res 9:782–787

SPSS/PC (1986) SPSS/PC for the IBM PC/XT/AT. SPSS Inc., Illinois

Tabatabai MA, Bremner JM (1969) Use of p-Nitrophenol phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tarafdar JC, Jungk A (1987) Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. Biol Fertil Soils 3:199–204

Tarafdar JC, Bareja M, Panwar J (2003) Efficiency of some phosphatase producing soil-fungi. Indian J Microbiol 43:27–32

Vassileva M, Vassilev N, Azcon R (1998) Rock phosphate solubilization by *Aspergillus niger* on olive cake based medium and its further application in a soil-plant system. World J Microbiol Biotechnol 14:281–284

Velazquez E, Rodriguez-Barrueco C (2007) First international meeting on microbial phosphate solubilization. In: Developments in Plant and Soil Sciences, vol 102. Springer, Salamanca

Wahid OAA, Mehana TA (2000) Impact of phosphate-solubilizing fungi on the yield and phosphorus-uptake by wheat and faba bean plants. Microbiol Res 155:221–227

Wakelin SA, Warren RA, Harvey PR, Ryder MH (2004) Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. Biol Fertil Soils 40:36–43